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Human lens lipids differ markedly from those of commonly used experimental animals

Jane M. Deeley[a], Todd W. Mitchell[b], Xiaojia Wei[a], John Korth[a], Jessica R. Nealon[a], Stephen J. Blanksby[a*] and Roger J.W. Truscott[c*]

School of Chemistry[a] and School of Health Sciences[b], University of Wollongong, NSW, 2522
and Save Sight Institute[c], University of Sydney, NSW, 2001, Australia

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*Author's to whom correspondence should be addressed:

Dr Stephen Blanksby, Department of Chemistry, University of Wollongong, NSW 2522, Australia, Tel: +61 2 4221 5484, Fax: +61 2 4221 4287, Email: blanksby@uow.edu.au

Professor Roger Truscott, Save Sight Institute, 8 Macquarie St, Sydney, NSW, 2001, Australia, Tel: +61 2 4221 3503, Fax: +61 2 4221 4287, Email: rjwt@eye.usyd.edu.au

SUMMARY

Electrospray ionisation tandem mass spectrometry has allowed the unambiguous identification and quantification of individual lens phospholipids in human and six animal models. Using this approach *ca*. 100 unique phospholipids have been characterized. Parallel analysis of the same lens extracts by a novel direct insertion-electron ionization technique found the cholesterol content of human lenses to be significantly higher (*ca.* 6 times) than lenses from the other animals.

The most abundant phospholipids in all the lenses examined were choline-containing phospholipids. In rat, mouse, sheep, cow, pig and chicken, these were present largely as phosphatidylcholines, in contrast 66% of total phospholipid in *Homo sapiens* was sphingomyelin, with the most abundant being dihydrosphingomyelins, in particular SM(d18:0/16:0) and SM(d18:0/24:1). The abundant glycerophospholipids within human lenses were found to be predominantly phosphatidylethanolamines and phosphatidylserines with surprisingly high concentrations of ether-linked alkyl chains identified in both classes. This study is the first to identify the phospholipid class (headgroup) and assign the constituent fatty acid(s) for each lipid molecule and to quantify individual lens phospholipids using internal standards. These data clearly indicate marked differences in the membrane lipid composition of the human lens compared to commonly used animal models and thus predict significant variation in the membrane properties of human lens fibre cells compared to those of other animals.

INTRODUCTION

The main body of the lens consists of concentric layers of slender, crescent shaped fibre cells, effectively creating a tightly packed arrangement of cell membranes. The main structural components of these cell membranes are integral membrane proteins, phospholipids and cholesterol. Phospholipids are a diverse range of molecules, consisting of a "head" group and one or two fatty acid "tails". While the "head" denotes the class of phospholipid (*e.g.*, phosphatidylethanolamine, phosphatidylcholine *etc.*) different combinations of fatty acid "tails" result in a myriad of possible molecules within each class.

It has previously been observed that the lipid composition of lens fibre cell membranes varies between animal species [1-4] and it has been suggested that phospholipid content may be correlated with maximum lifespan [5]. Human and primate lenses have been found to be rich in dihydrosphingomyelins, with these phospholipids comprising approximately 50% of total phospholipid in humans [6, 7]. Dihydrosphingomyelins are present as only minor constituents in non-primate lenses with the major phospholipid classes in shorter-lived animals being mostly phosphatidylcholine, sphingomyelin and phosphatidylethanolamine [2, 3, 8].

Previous investigations of lens cell membrane composition typically utilized techniques such as ^{31}Phosphorus nuclear magnetic resonance (^{31}P NMR) spectroscopy [2, 9-11] to analyse the phospholipid "heads". This technique resolves phospholipids based on their head-groups alone and provides little information on the fatty acid composition. Gas chromatography has been used to examine the fatty acid "tails" [4] and this involves initial hydrolysis of the lipids followed by derivatisation. Experiments using gas-chromatography have shown that human lenses contain largely palmitic acid and a monounsaturated 24-carbon chain fatty acid [12, 13], whilst in bovine and rabbit lenses a monounsaturated 18-carbon chain fatty acid predominated [14]. Thin-Layer chromatography has been used in conjunction with gas chromatography in order to identify the fatty acids associated with a particular class of phospholipids in rabbit lenses [8] but such studies do not characterize the molecular structure of individual lipids.

Matrix-assisted laser desorption ionization mass spectrometry (MALDI) has also been used for the characterisation of phospholipids in porcine [15], bovine [16] and human [17] lenses. Analysis of phospholipid mixtures is difficult using MALDI as there are significant differences in the ionisation efficiencies of different classes [18] and thus only relative quantitation has been achieved [15]. In the current study the use of internal standards for each phospholipid class enabled quantitation of all molecules detected by allowing for the differences in ionisation efficiency. Experiments using MALDI deduced that there are approximately 80 different phospholipids in porcine lenses [15], however they were not capable of distinguishing between isobaric phospholipids because identification was based solely upon the mass-to-charge ratio (*m/z*) of the parent phospholipid [15]. MALDI provided only the total acyl chain composition of each phospholipid (*e.g.,* 36:2) and therefore complete characterisation of individual phospholipids at a molecular level was not possible (*i.e.,* 36:2 may be 18:1/18:1 or 18:0/18:2) [15]. By contrast a triple-quadrupole mass spectrometer, capable of neutral loss, precursor ion and fragment ion scans, such as the one used in this study, can be used to unambiguously identify the molecular composition of glycerophospholipids and sphingolipids in a tissue extract.

"Shotgun lipidomics" is a contemporary, electrospray-ionisation tandem mass spectrometry (ESI-MS/MS) based method that can be used for the complete characterization of phospholipids in lipid extracts [19]. This method is capable of not only providing quantitative information on lipid classes, but also precise information on the fatty acid composition of each individual phospholipid [20, 21]. In this study ESI-MS/MS was employed to provide quantitative data on the phospholipid composition of lenses from several animals. In addition, a novel direct insertion electron ionisation mass spectrometry (DI/EI-MS) method was developed and implemented for the rapid quantification of free cholesterol in the same lipid extracts. Electrospray ionisation of cholesterol is generally inefficient in crude lipid extracts due to the presence of more readily ionisable phospholipids. While this limitation can be overcome by fractionation, the method presented here can be used in parallel with the electrospray analysis and does not require time-consuming chromatographic separation of the extract.

Human lenses are difficult to source reliably, so it is understandable that animal tissues are often used as models for the study of human lens disorders. Rodent lenses are the most commonly used [22-29]; however investigators have also used pig [30-33], sheep [34, 35], cow [36-38] and chicken [39-41]. One purpose of this study was to characterise the membrane composition of lenses from humans as well as those from commonly used experimental animals in order to discover how close the membranes of animal lenses are to humans, and in this way provide information as to the most appropriate animal model for the human lens. The current study is the first to identify the fatty acids associated with individual phospholipids in the human lens and also the lenses of commonly used experimental animals. This novel information provides valuable new insight into the biophysical and biochemical properties of the lens fibre cell membranes in these animals.

MATERIALS AND METHODS

Materials

All organic solvents used were HPLC grade and purchased from Crown Scientific (Moorebank, Australia). Analytical grade butylated hydroxytoluene (BHT) was purchased from Sigma Aldrich (Castle Hill, Australia). Phospholipid standards were synthesised by Avanti Polar Lipids (Alabaster, USA) and purchased from Auspep (Parkville, Australia) with the exception of GPCho (20:0/20:0) that was purchased from Sigma Aldrich (Castle Hill, Australia).

Lenses

Eyes were obtained from the following sources in N.S.W., Australia : Bovine, Wollondilly Abattoirs, Picton; Porcine, Crownpork, Strathfield; Ovine, Milton District Meats, Milton; Chick, Inghams, Hoxton Park. Male C57bl/6 mice were obtained from the Animal Resource Centre (Perth, Australia), kept under standard 12:12 light: dark conditions and euthanized by exposure to carbon dioxide. Male Sprague-Dawley rats were obtained from Animal Resource Centre (Perth, Australia), kept under standard 12:12 light: dark conditions and euthanized by peritoneal injection of sodium pentobarbitone at a concentration of 0.6 mg/g body weight. Lenses were removed from mouse, rat, cow, pig, sheep and chick eyes within 6 hours of death and stored at -80 °C until required. All animal experiments conducted were approved by the animal research ethics committee at the University of Wollongong. Human lenses were collected from eyes donated to the New South Wales Lions Eye Bank at the Sydney Eye Hospital and included three 60 year old females and one 60 year old male. These tissues were stored at -80 °C until required. This work was approved by the human research ethics committees at the University of Sydney (# 7292) and the University of Wollongong (HE 99/001). Four lipid extracts were obtained and analysed from lenses from each of the 7 species examined in this study (*i.e.,* n=4). For rat, chick, cow, pig, sheep and human, each lipid extract was obtained from a single lens. In human, each of the four lenses studied was from a different human subject. For mouse, five lenses were pooled to provide sufficient tissue for each of the four lipid extracts (*i.e.,* a total of 20 mouse lenses). For each of the seven species the data was analysed and the quantity of individual phospholipids was averaged for the four extracts and the standard error of the mean was calculated (n=4).

Nomenclature

In this paper glycerophospholipids and sphingolipids will have the abbreviations of Fahy *et al.*[42]. 1-*O*-alkyl ether and 1-*O*-alkenyl ether lipids will have the abbreviations employed by Zemski Berry *et al.* [43]. Cholesterol and deuterated cholesterol will have the abbreviations (CHOL) and ($CHOL_{D6}$), respectively.

Lipid Extraction

Lenses were frozen under liquid nitrogen, ground using a mortar and pestle and chloroform:methanol (2:1 v/v) containing 0.01% BHT was added to the lens material at a ratio of 20:1 solvent to tissue (v/w). A methanolic internal standard solution was prepared comprising GPCho (19:0/19:0), 95 μM; SM (d18:0/12:0), 84 μM; GPSer (17:0/17:0), 34 μM; GPEtn (17:0/17:0), 39 μM; GPGro (17:0/17:0), 25 μM, GPA (17:0/17:0), 25 μM. The concentration of each internal standard was optimised to reflect the different concentrations of lipid classes within the lens extracts. The internal standard solution was prepared by appropriate dilution of individual stock solutions where the concentration of each had been independently established by phosphorus assay [44]. The internal standard mixture was added to the homogenates at 1400 $\mu L.g^{-1}$(tissue) for later quantification of phospholipids. The homogenates and solvents (including internal standards) were rotated overnight and standard procedures to obtain the lipid extract were followed thereafter as described by Folch *et al.* [45] with the exception that sodium chloride was substituted with aqueous ammonium acetate (0.15 M). Total phospholipid concentration of each crude lipid extract was determined by phosphorus assay [44] and samples were stored at -80 ºC until analysed.

Mass Spectrometry

All mass spectra were obtained using a Waters QuattroMicro™ (Waters, Manchester, U.K.) equipped with a z-spray electrospray ion source and controlled by Micromass Masslynx version 4.0 software. Capillary voltage was set to 3000 V, source temperature 80 °C and desolvation temperature 120 °C. Cone voltage was set to 50 V and 35 V in negative and positive ion modes, respectively. Nitrogen was used as the drying gas at a flow rate of 320 $L.h^{-1}$. Phospholipid extracts were diluted to a final concentration of 40 μM with the addition of methanol:chloroform (2:1 v/v). Samples were infused into the electrospray ion source at a flow rate of 10 $\mu L.min^{-1}$ using the instrument's on-board syringe pump. In all precursor ion, neutral loss and product ion scans, argon was used as the collision gas at a pressure of 3 mTorr and the collision energy was set between 22-50eV depending upon the scan being performed (see Supplementary Material Table S1). GPA, GPGro, GPEtn, GPSer and GPIns form $[M-H]^-$ anions in negative ion mode and were detected in the mass range of *m/z* 640-940. SM and GPCho were detected in positive ion mode at *m/z* 640-850. GPEtn and GPSer also form abundant $[M+H]^+$ ions in positive mode that were used for quantification of these two classes. The formation of these ions was enhanced, in some instances, by the addition aqueous ammonium acetate (1 M) [46]. A combination of precursor and neutral loss scans were used to identify the headgroup of each phospholipid in positive and negative ion modes as previously demonstrated [47]. For phospholipid classes observed in negative ion mode, precursor ion scans of the fatty acid carboxylate anions were used for the identification of the fatty acids coupled with each individual phospholipid. For the identification of GPCho-coupled fatty acids, neutral loss scans for the loss of lithiated fatty acids were performed in positive ion mode after the addition of aqueous lithium acetate to the lipid extract (to a final concentration of 200 μM of lithium acetate). To distinguish between SM(d18:1) and SM(d18:0), neutral loss scans specific for the sphinganine (431.2 Da) or sphingosine (429.2 Da) backbone, respectively, were performed in the presence of lithium acetate [48]. The identification of suspected GPEtn 1-*O*-alkenyl (plasmenyl) ether phospholipids was confirmed by product ion scans of the $[M+H]^+$ molecular ion that revealed characteristic fragment ions for the alkenyl ether linked carbon chain and the ester linked carbon chain [43]. These spectra also revealed a characteristically low abundance of the fragment ion resulting from the neutral loss of 141 Da as previously reported [43]. A full record of all scans performed in this study, including the collision energy employed, is provided as supplementary information (see Table S1).

Where two or more isomeric phospholipids were identified, a product ion spectrum was obtained and the relative abundance of each isomer was determined from comparison of the combined abundances of the two fatty acid carboxylate ions arising from each lipid with the combined peak area of all carboxylate anions present. Neither the relative position of the acyl chains on the glycerol backbone [49] (often called the *sn*-position) nor the position of double bonds [50, 51] can be rigorously assigned from these data and therefore discussions in this paper are based on the naturally abundant regioisomers only.

For phospholipid quantification, headgroup-specific neutral loss and precursor ion mass spectra were obtained from averaging a minimum of 100 scans. Phospholipids were quantified by comparing their peak areas with the appropriate internal standard for each class after correction for isotope contributions (see below) [19]. Although there exist differing reports in the literature, some authors have suggested a decreasing detection efficiency for phospholipids with increasing acyl chain length and have thus recommended the use of two or more internal standards for each phospholipid class [47, 52, 53]. Examination of this effect was carried out under our experimental conditions for the representative *m/z* 184 precursor ion scan used for quantification of phosphatidylcholines. A mixture of the phospholipid standards GPCho (15:0/15:0), GPCho (17:0/17:0), GPCho (19:0/19:0) and GPCho (20:0/20:0) was prepared at concentrations ranging from 20-160 μM (total lipid) by quantitative dilution of stock solutions of each lipid where the concentration had been independently verified by phosphorus assay [44]. Precursor ion scans of these standard solutions revealed no change in detection efficiency with acyl chain length across the mass (*m/z* 706-846) and concentration (20-160 µM total lipid) ranges used in this study (these data are provided as supplementary material, Figure S1). These observations are supported by several other studies, that have also shown no trend in detection efficiency at total phospholipid concentrations below 50 µM [19, 54, 55] as used here. Thus in the present study we employed a single internal standard for quantification of each phospholipid class in-line with literature precedent [47, 55-58].

Isotope corrections were used to correct for; (i) the greater contribution of isotopic ions to the total abundance of larger phospholipids, and (ii) the contribution of isotope peaks of one phospholipid to the area of the monoisotopic peak of a larger one. To achieve this, the isotopic ion distribution of each phospholipid was calculated from isotope models and the area of the monoisotopic peak multiplied by the calculated correction factor. This calculation started with the smallest observed phospholipid so that any contribution of its isotope peaks to the area of phospholipids of greater *m/z* could be subtracted before subsequent isotope calculations were performed.

An approximate quantification of GPIns was achieved via comparison of the abundance of the $[M\text{-}H]^-$ molecular ion with that of the GPGro (17:0/17:0) internal standard in negative ion mode. An empirical correction factor of 0.75 was applied to account for the differences in relative ionisation efficiencies of the two head groups [53]. A correction factor of 3.4 was also employed for the quantification of GPEtn 1-*O*-alkenyl to account for the different fragmentation efficiency of these ethers compared with diacyl GPEtn in forming the neutral loss of 141 Da [58].

It has been suggested that inherent limitations of the shotgun approach, mostly associated with ion suppression effects (and lipid-lipid interactions at high concentrations), make it a semi-quantitative method with estimations of experimental uncertainty as high as 5% [19]. Comparison of lipid profiles acquired under the same conditions, as carried out in this experiment however, are expected to significantly reduce the associated uncertainty through cancellation of systematic errors.

Mass Spectrometry of Cholesterol

A Shimadzu™ QP5050 GC-MS was used for cholesterol quantification by direct-insertion electron-impact mass spectrometry (DI/EI-MS). A direct insertion probe was used to bypass the gas chromatograph. Approximately 1 µL of sample was drawn into the open end of a heat-sealed glass capillary tube, allowed to dry at room temperature before direct insertion into the mass spectrometer. A temperature program was chosen to heat the probe to 200 ºC and provide optimal thermal desorption of the analytes. Deuterated cholesterol ($CHOL_{D6}$) was used as the standard to generate a standard curve, prepared by using cholesterol (CHOL) and $CHOL_{D6}$ in mass ratios ranging from 0.1 to 3.0 CHOL/$CHOL_{D6}$. Selected ion monitoring (CHOL: *m/*z 386, 353; $CHOL_{D6}$: *m/z* 392, 359) was used to generate the standard curve and to quantify cholesterol for each sample. Lipid extracts from mouse, rat, pig, cow, sheep, chick and human lenses were used in the study. $CHOL_{D6}$ was added to each of the lipid extracts and ion intensity ratios of the ions listed above were used with the standard curve to determine the concentration of cholesterol in the lens lipid extracts.

RESULTS

1. Lens Phospholipids

All phospholipids detected by ESI-MS/MS in lenses from the seven species were identified and quantified and the full data set is provided as supplementary material (Table S2-6). These data are summarised in Figure 1, which shows the content of all classes of glycerophospholipid and sphingomyelin in the lenses of each of the seven species investigated. Figure 1 illustrates the unique phospholipid composition for each animal species and shows the animals most similar in phospholipid class distributions are sheep, cow and pig. The most abundant phospholipids identified in all lenses were choline-containing, namely phosphatidylcholine (GPCho), sphingomyelins (SM(d18:1)) and dihydrosphingomyelins (SM(d18:0)). GPCho dominated the phospholipid profiles of all of the non-human animals, ranging from 410-950 nmol.g(tissue)$^{-1}$, representing 31-46 % of total phospholipids. GPCho was found to have the lowest concentration in man (370 nmol.g(tissue)$^{-}$) representing only 11% of total human lens phospholipids. In particular, the GPCho concentration in mouse and rat lenses were more than double that found in human. Of the other phospholipid classes sphingomyelin was also abundant, contributing between 280-390 nmol.g(tissue)$^{-1}$ in all the non-human mammals. GPEtn and GPSer also contributed significantly accounting for a combined *ca.* 37% of the total phospholipid pool. In all of the animal lenses, phosphatidic acid (GPA) and phosphatidylinositol (GPIns) were minor constituents contributing a combined total of less than 6% of total phospholipids.

The human lens was distinctly different from all of the other animals, with dihydrosphingomyelins alone contributing 1640 nmol.g(tissue)$^{-1}$. This represents almost half of total phospholipid in the human lens and almost double the concentration of any other lipid class in any of the other six species. Although the proportion of GPEtn in man appears to be within the range of the other animals (Figure 1), a detailed examination revealed that the major phospholipids in this class were 1-*O*-alkyl ethers distinct from the abundant diacyl GPEtn identified in all other animals except chick. These trends are discussed in more detail below.

[Figure 1]

2. Choline-containing lipids

Choline-containing phospholipids are known to be the most abundant phospholipids in mammals [59]. Phosphatidylcholine, sphingomyelin and dihydrosphingomyelin were identified in the lens extracts using precursor ion scans for *m/z* 184 in positive ion mode. Representative mass spectra for each of the seven species are presented in Figure 2. A qualitative examination of these raw data suggests that although there are some differences between the non-human animals, they appear to contain the same major lipid molecules. These spectra are dominated by ions at *m/z* 706, 734 and 760 that were assigned by these and complementary mass spectra (see Materials and Methods) to GPCho (14:0/16:0), (16:0/16:0) and (16:0/18:1), respectively. Figure 2 also shows that the human lens differs markedly in that the most abundant choline-bearing lipid is observed at *m/z* 705 and is assigned to the completely saturated dihydrosphingomyelin SM (d18:0/16:0). Indeed, this difference is underrepresented in the raw data (Fig. 2) due to the significantly lower fragmentation efficiency of sphingomyelins in forming the *m/z* 184 ion compared to GPCho. Once quantification of sphingomyelins was achieved using a class-specific internal standard (SM(d18:0/12:0), *m/z* 649 highlighted in Fig. 2) the dramatic difference in dihydrosphingomyelin concentration became clearly evident as highlighted in Figure 1.

[Figure 2]

2a. Phosphatidylcholines

GPCho (16:0/16:0) was found to be one of the two most abundant phosphatidylcholines in all the animal lenses, with the exception of human (Table 1). In humans, the monounsaturated GPCho (16:0/18:1) was the most abundant contributing over 33% of all GPCho lipids. The most abundant GPCho constituents in all animal species were found to be quite homogeneous, being composed of just two fatty acids, 16:0 and 18:1.

[Table 1]

2b. Sphingolipids

Sphingolipids in the lenses of all species, with the exception of human, were found a relatively narrow concentration range of 300-470 nmol.g(tissue)$^{-1}$ representing 15-29% of total phospholipid. In contrast the human lens was found to contain 2300 nmol.g(tissue)$^{-1}$ of sphingomyelins representing almost 70% of all lens phospholipids. In general, the sphingolipids, sphingomyelin and dihydrosphingomyelin, differ from glycerophospholipids by having only one carbon chain linked (via an amide bond) to the sphingosine, or sphinganine backbone, respectively. In this study the identity of sphingomyelins was determined using the method of Hsu and Turk, which involves neutral loss scans for 429 and 431 Da to identify lithiated sphingomyelin and dihydrosphingomyelin, respectively [48]. In order to confirm the characterisation of the most abundant human lens phospholipids, fragment ion spectra of $[M+Li]^+$ were obtained to show evidence of the sphingosine (*m/z* 264) or sphinganine (*m/z* 266) long-chain base. In the animal and human lenses in this study SM(d18:1) and SM(d18:0) were generally found to have associated carbon chains of 16:0 and 24:1. Table 1 shows that the single most abundant lipid in human lenses, which contributed >25% of all the human lens phospholipids, was the completely saturated SM(d18:0/16:0). This has previously been identified by Byrdwell *et al.* as the most abundant phospholipid in the human lens [60]. Dihydrosphingomyelins were detected in the other animals however only in trace amounts <3%

with the exception of chicken in which this class contributed a significant amount to its lens phospholipid profile (11%).

3. Phosphatidylethanolamines

Phosphatidylethanolamines were identified in the lens extracts using neutral loss scans for 141 Da in positive ion mode. Representative mass spectra for each of the seven species are presented in Figure 3. A qualitative examination of these spectra reveal a larger variation in fatty acid composition than observed for GPCho (Fig. 2), or indeed any of the other glycerophospholipid classes (Fig 5,6, Table 1). These raw data (Fig. 3) suggest similarities between non-human mammals and some congruence in the GPEtn composition of human and chick. The most abundant ion in the spectra of non-human mammals was *m/z* 744 which based on these spectra and complementary spectra was assigned as GPEtn (18:1/18:1). This molecule was found to contribute 26-55% of total GPEtn in mouse, cow rat, pig and sheep.

[Figure 3]

The most abundant ion in the 141 Da neutral loss spectra of chick and human was observed at *m/z* 730. A fragment ion spectrum in negative ion mode of the corresponding *m/z* 728 $[M-H]^-$ anion revealed a single fatty acid carboxylate anion at *m/z* 281. The observation of a lone radyl in this spectrum is consistent with an ether linkage at the *sn*-1 position on the glycerol backbone. Based on these data the lipid could be assigned as either an 1-*O*-alkenyl (plasmenyl) ether or an 1-*O*-alkyl (plasmanyl) ether. To confirm the structural assignment, fragment ion spectra were obtained for the $[M+H]^+$ ion of a range of suspected ether linked GPEtn. The fragment ion spectrum (Figure 4a) of the closely related GPEtn at *m/z* 728 (taken from pig) shows a fragment ion at *m/z* 339 indicative of a 1-*O*-alkenyl ether-linked monounsaturated 18-carbon chain. This confirms the identity of the ion at *m/z* 728 as GPEtn (18:1p/18:1). The fragment ion spectrum of the $[M+H]^+$ at *m/z* 730 is shown in Figure 4b and does not conform with the previously reported behaviour of phosphatidylethanolamines containing an 1-*O*-alkenyl ether linkage, notably no fragment ion corresponding to the *sn*-1 radyl is observed [43]. In an independent study using new technology, developed in our laboratory, this ion was definitively assigned to the structure of 1-*O*-alkyl ether GPEtn (18:1e/18:1) [51]. An analogous investigation of the structure of *m/z* 704 in human identified this lipid as GPEtn (16:0e/18:1).

[Figure 4]

GPEtn 1-*O*-alkyl ethers made up a large proportion (50%) of the total GPEtn in humans with the (18:1e/18:1) lipid alone contributing more than 40% of total GPEtn (Table 1). Similarly, in the chick lens, GPEtn (18:1e/18:1) was found to contribute more than 20% of phospholipids in this class. In contrast 1-*O*-alkenyl ethers, also known as plasmalogens, were significant contributors to the total GPEtn pool in rat and mouse where GPEtn (18:1p/18:1) was one of the two most abundant phosphatidylethanolamines with relative abundances of 21 and 11% (total GPEtn), respectively.

4. Phosphatidylserines

Phosphatidylserines were identified in the lens extracts using neutral loss scans of 185 Da in positive ion mode. The corresponding lipids were also identified in negative ion mode using neutral loss scans for 87 Da thus providing additional support for their assignment as phosphatidylserines (data not shown). Representative mass spectra for each of the seven species are presented in Figure 5. A qualitative examination of these raw data suggests that human is significantly different from other animals. Indeed, the most abundant phosphatidylserine present in human, observed at *m/z* 748, is completely absent in cow, sheep, rat and mouse, and of negligible abundance in chick and pig. In other animals, the most consistently abundant GPSer are observed at *m/z* 788 and 790 which were identified by this and complementary scans as diacyl GPSer (18:0/18:1) and (18:1/18:1). These molecules contributed 17-55 and 15-33 % of total GPSer, respectively, in all the animal lenses (Table 1).

A fragment ion spectrum in negative ion mode of *m/z* 746 was obtained from a human lens extract. This ion, the conjugate $[M-H]^-$ anion of the *m/z* 748 cation found to be abundant in human, revealed a single fatty acid carboxylate anion at *m/z* 281 and the notable absence of a fragment ion at *m/z* 153. The observation of a lone radyl in this spectrum and the absence of the glycerophosphate marker [47], is consistent with an ether linkage at the *sn*-1 position. Based on these data the lipid was assigned as an 1-*O*-alkyl (plasmanyl) ether, which was further supported by the unaltered abundance of these ions in the presence of strong acid excluding the possibility of an 1-*O*-alkenyl (plasmenyl) ether linkage in the molecule. Although unusual, there is precedent for the presence of these 1-*O*-alkyl phosphatidylserines in animal tissues such as rat lung [61]. The observation of 1-*O*-alkyl GPSer in the human lens is also consistent with the appearance of the analogous 1-*O*-alkyl ether GPEtn in the same tissue (see above). In humans, such GPSer 1-*O*-alkyl ethers were found to be the most abundant GPSer molecules with the combination of the ethers identified at *m/z* 748, 774 and 776 (Figure 5) contributing >60% of GPSer in human lens. GPSer 1-*O*-alkyl ethers were also present in the chicken and to a lesser extent in pig (e.g., *m/z* 748 Figure 5), but were present at much lower levels compared to analogous ethers in GPEtn.

[Figure 5]

5. Phosphatidic acid, phosphatidylglycerol and phosphatidylinositol

Figure 6 shows representative precursor ion scans of *m/z* 153 for all seven species which were used to identify diacyl glycerophospholipids in the following classes; GPA, GPGro, GPIns and GPSer. These data reveal low abundances of both GPA and GPIns, with no significant amount of GPGro, in all lenses (Figure 6). Quantification of GPA and GPIns revealed that these classes contribute less than 150 nmol/g(tissue) and together represent <6% total phospholipids in any species investigated. In human and all animal lenses, except rat, GPIns (18:0/20:4) was the most abundant GPIns.

[Figure 6]

6. Cholesterol

A clear difference in the lens cholesterol concentration between humans and the other animals analysed can be seen in Figure 7. None of the animal lenses had a cholesterol concentration greater than 1 mg.g(tissue)$^{-1}$ whereas the human lens contained 6.4 mg.g(tissue)$^{-1}$.

[Figure 7]

DISCUSSION

Through the use of ESI-MS/MS, this study provides the first comprehensive identification and quantification of more than 100 lens phospholipids across seven animal species. This allows the first opportunity for direct interspecies comparison of lens phospholipids at this detailed level. The data show the lens membrane lipids of the seven animals examined to be quite distinct with the human lens lipid profile unique as highlighted in Figure 8. Specific features of the lenses of the seven animals that were investigated are discussed below:

[Figure 8]

1. Mouse

In terms of percentage, the mouse lens was the most GPCho-rich of all the lenses analysed (46% of total phospholipid) and it also it had the smallest relative amount of sphingomyelins (15%). Phosphatidylcholines were dominated by the completely saturated GPCho (16:0/16:0) which is in accord with previous work [46]. A previous study of phospholipids using ^{31}P NMR suggested that phosphatidylethanolamines are the most abundant phospholipids in mouse lens (30 mol %) followed by phosphatidylcholines (21.5%) and sphingomyelins (20.5%) [2]. This is at odds with the current study, however, the significant contribution of unknowns to the total phospholipid composition of the lens in the previous study may account for some of this discrepancy.

2. Rat

Our finding that the rat contained 41% total PC and 16% SM (d18:1) is in agreement with previous studies which have reported rat lenses as having between 31-43% GPCho and only 6-16% SM(d18:1) [2, 3, 62]. The SM(d18:1) were mostly comprised of short chain saturated (16:0) and long chain (24:1) fatty acids.

3. Pig

Pig lenses were similar in overall lipid composition to cow and sheep (Figs 1 and 8). This is not surprising owing to their shared taxonomy, i.e., all are even-toed ungulates.

Using a protracted series of techniques Byrdwell has previously identified a broad range of sphingomyelins in the pig lens [63] approximately half of which were also identified in the current study of the entire phospholipidome. Data from the present study shows the pig lens to contain 26% SM (d18:1) which is in accord with previous studies showing SM(d18:1) to contribute 21-31% of pig lens phospholipids [2, 3, 64]. Dihydrosphingomyelins were detected in small amounts in pig lenses with this class contributing 3% of total phospholipids and 10% of total sphingolipids which is in agreement with previous HPLC studies reporting dihydrosphingomyelin at 14% of sphingolipids [63]. At the molecular level the most abundant sphingomyelin and dihydrosphingomyelin were found to be SM (d18:1/16:0) [15, 63] and (d18:0/16:0) [63] which is also in agreement with literature precedent.

In previous studies GPEtn had been found to contribute 24-34% of total porcine lipids [2, 3]. This is in contrast to the present study where all GPEtn lipids contributed 12% of total lipids. Two recent studies described GPEtn 1-*O*-alkenyl ethers as being major lipids in porcine lenses (*e.g.,* 12.2% of total phospholipids) [2, 15]. In our study we found alkenyl ether-linked lipids to contribute 22% of GPEtn species and therefore 2.6 % of total pig lens phospholipids.

4. Sheep

Published data on sheep lens lipids vary considerably. One study reported 30.5% SM(d18:1) and 20 % GPCho, [3] whereas a later paper listed 14.3% SM(d18:1) and 33% GPCho [2]. Our results of 20% SM (d18:1) and 37% GPCho are closest to the study by Iwata *et al.*[2] The present study detected GPEtn as 22% of total phospholipids, which is in broad agreement with previous studies where GPEtn was found to contribute 29-33 % of total phospholipids [2, 3].

5. Cow

Bovine lenses had the largest proportion of SM(d18:1), but appeared not unlike the sheep and pig in terms of the overall distribution of the various phospholipid classes in both absolute and relative terms (Figs 2 and 8). In bovine lenses it has previously been reported that the major phospholipids contain the saturated palmitic acid and monounsaturated oleic acid, contributing 33 and 34% of total fatty acids respectively [1]. This is in agreement with the present study with one or both of the two major phospholipids in each class containing either 16:0 or 18:1. Early studies described phosphatidylethanolamine, including the ether-linked variants, to be major phospholipids in the bovine lens with reports of GPEtn comprising 34% [3] and 28.6% [2] total phospholipids. This is in broad agreement with the present study in which bovine lenses were found to contain GPEtn at 22% with 1-*O*-alkenyl ethers contributing 12.6% of total GPEtn and almost 3% of total phospholipids. Consistent with the results of this investigation, phosphatidylcholine and sphingomyelin have been reported to be the major classes of phospholipids in the bovine lens [5].

6. Chick

The bird lens contained the highest absolute (ca. 600 nmol/g) and relative (26% of total phospholipids) amount of GPEtn of all the animals. Significant amounts of dihydrosphingomyelins were also present with these molecules contributing 11% of total phospholipids and 55% of total sphingolipids. The fatty acid composition was similar to that of human dihydrosphingomyelin with the major lipids present being SM (d18:0/16:0) and (d18:0/24:1). Of most interest, chick lenses appear to contain the same major ether-linked fatty acids in GPEtn that are found in humans. The two most abundant classes in the chick lens were found to be GPCho and GPEtn, while previous reports suggest chicken lenses contain sphingomyelin and phosphatidylethanolamine as the most abundant phospholipid classes [3].

7. Human

Human lenses are dominated by dihydrosphingomyelins, with SM (d18:0/16:0) being the most abundant phospholipid. This unusual phospholipid was first identified in the lens by Byrdwell and colleagues [7]. Dihydrosphingomyelin was found to be of similar abundance in teenage lenses (data not shown), suggesting that the abundance of this lipid class is not simply a function of age. The fatty acid composition of the two major sphingomyelins, SM (d18:1/16:0) and SM (d18:1/24:1) was the same as the dihydrosophingomyelins, which is in agreement with a previous report [60]. This is not surprising as the divergence between SM (d18:1) and SM (d18:0) synthetic pathways occurs after ceramide synthase has attached the fatty amide [65]. Nevertheless, the elevated levels of dihydrosphingomyelins in human lenses suggest a preference for the SM (d18:0) pathway, a situation that is theoretically compatible with a high cell oxidation state [65]. In concert with the current findings, the most abundant SM (d18:0) in human lens membranes have previously been reported to be SM (d18:0/16:0) and SM (d18:0/24:1) that contributed 57.8 and 23.3% of dihydrosphingomyelins respectively [60]. In the current study GPCho was found to represent only 11% of total phospholipids compared to a minimum of 28% in the other animals studied. This finding is supported by previous studies that indicate GPCho in human lenses is only a minor phospholipid class (< 5% of total phospholipids) [2, 3, 9, 66].

Examination of phospholipid-bound fatty acids in the current investigation found the most abundant fatty acids to be 16:0, 18:0, 18:1 and 24:1 in accord with most previous studies of fatty acid composition in the human lens [4, 12, 13, 67]. One study, however, has reported that long chain fatty acids (25-26 carbons) contribute almost 25% of all fatty acids in the human lens [68] . No evidence for these very long chain lipids was uncovered in this investigation or other studies of fatty acid composition [4, 12, 13, 67].

Also distinct from the other six species, human GPEtn and GPSer were both chiefly composed of ether-linked lipids. GPEtn (18:1e/18:1) was found to be the most abundant glycerophospholipid, and the fourth most abundant phospholipid overall in the human lens. The 1-*O*-alkyl ethers in GPEtn differ only in the head group to the GPSer ethers and may provide evidence for a precursor-product relationship in agreement with the literature, *i.e.*, GPEtn can be formed through decarboxylation of GPSer [69]. It is likely that the unusual GPEtn that we have described in human lenses, is the same as that documented previously as "PE-related" using ^{31}P NMR [9]. Huang *et al.* were not able to completely identify these unusual phospholipids which were found to contribute between 27-34% of clear human lenses [9]. It was postulated, however, that they were not 1-*O*-alkenyl ethers [9].

The free cholesterol content of all lenses was also measured using a novel direct-insertion electron-ionisation mass spectrometric assay employing deuterated cholesterol as internal standard. The concentration in the non-primate lenses was quite constant across the species. In bird lenses the cholesterol appeared to be lower (mg/gram wet weight) than in mammals but this is likely to be a reflection of the higher water content of chicken lenses. Once again human lenses were unique in containing very high levels of cholesterol. Cholesterol in the human lens has been reported as 5.7 mg/g in the cortex and 5.9 mg/g in the nuclear region [70], which is in broad agreement with our results. Previous research has shown bovine lenses to contain 0.67 - 0.92 mg/g [70], which is also in accord with our results.

We can only speculate about the reasons for the significant differences that we observed in the lens lipids of the seven animal species. Nonetheless, such significant differences in membrane composition are likely to be associated with major differences in the biochemical and biophysical behaviour of the membranes. Membrane lipids represent only a part of the lens, and the protein content and crystallin composition, as well as the complement of enzymes, also differ significantly in lenses from different species. The ratio of protein to lipid has been found to be 2.89 in humans lenses, whilst in cow and chicken it is 1.52 and 1.29 respectively [71, 72]. It appears that the lens is able to tolerate quite a wide range of membrane phospholipid and cholesterol content.

It is clear that human lenses are quite different from those of commonly used animal models in terms of membrane lipid composition, and such differences need to be borne in mind when experimental data obtained from laboratory animals are used to make predictions about the properties of human lenses, particularly in relation to common clinical conditions such as presbyopia and cataract.

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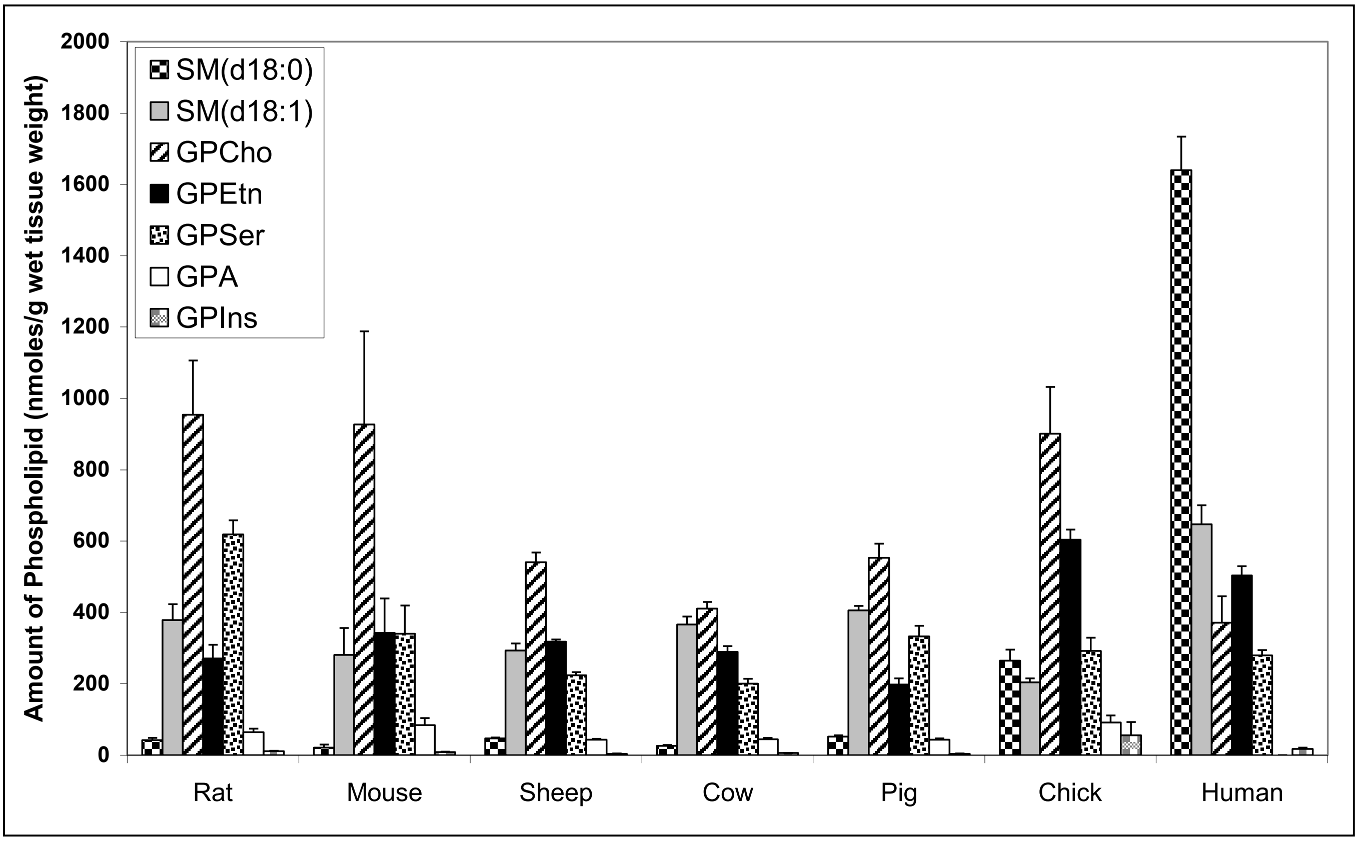
Figure 1



SM(d18:0)
SM(d18:1)
GPCho
GPEtn
GPSer
GPA
GPIns
Amount of Phospholipid (nmoles/g wet tissue weight)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Rat
Mouse
Sheep
Cow
Pig
Chick
Human

Figure 2

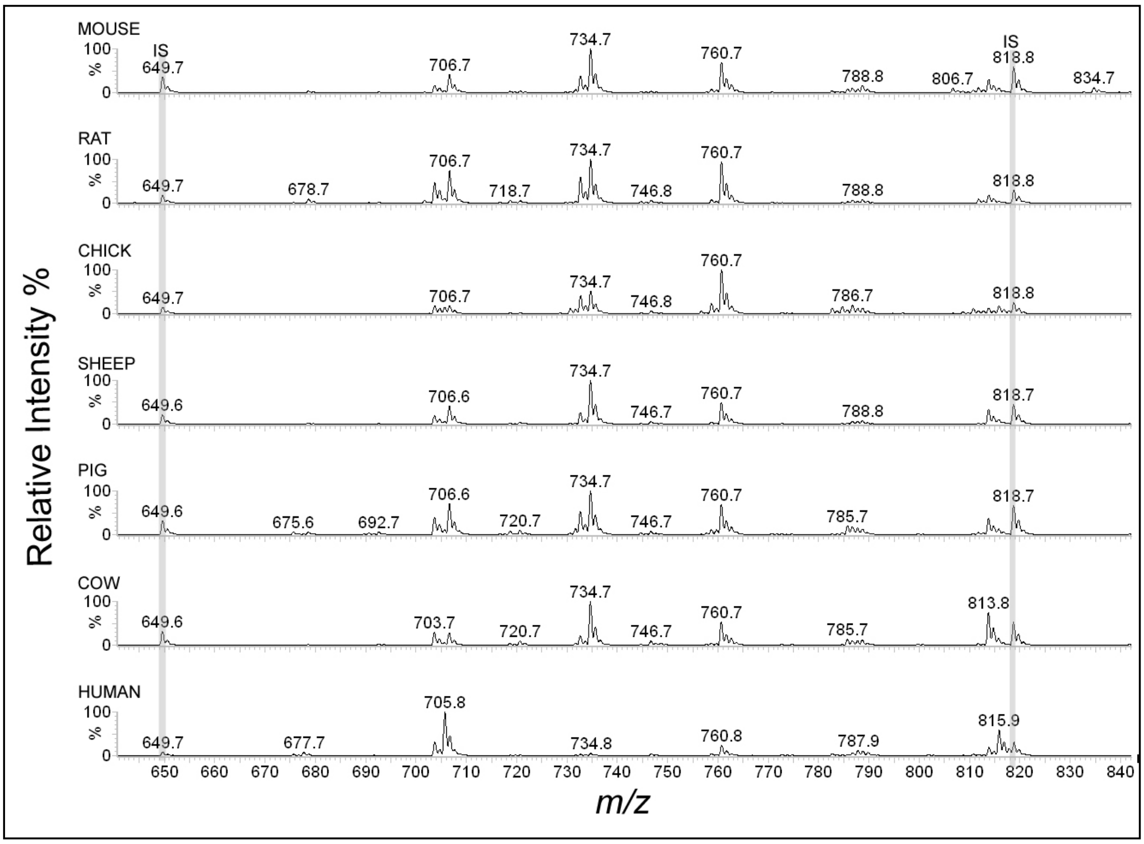

MOUSE
RAT
CHICK
SHEEP
PIG
COW
HUMAN
IS
649.7
706.7
734.7
760.7
788.8
806.7
818.8
834.7
678.7
718.7
746.8
786.7
649.6
706.6
746.7
675.6
692.7
720.7
785.7
818.7
703.7
813.8
705.8
677.7
734.8
760.8
787.9
815.9
100
%
0
650
660
670
680
690
700
710
720
730
740
750
760
770
780
790
800
810
820
830
840
Relative Intensity %
m/z

Figure 3

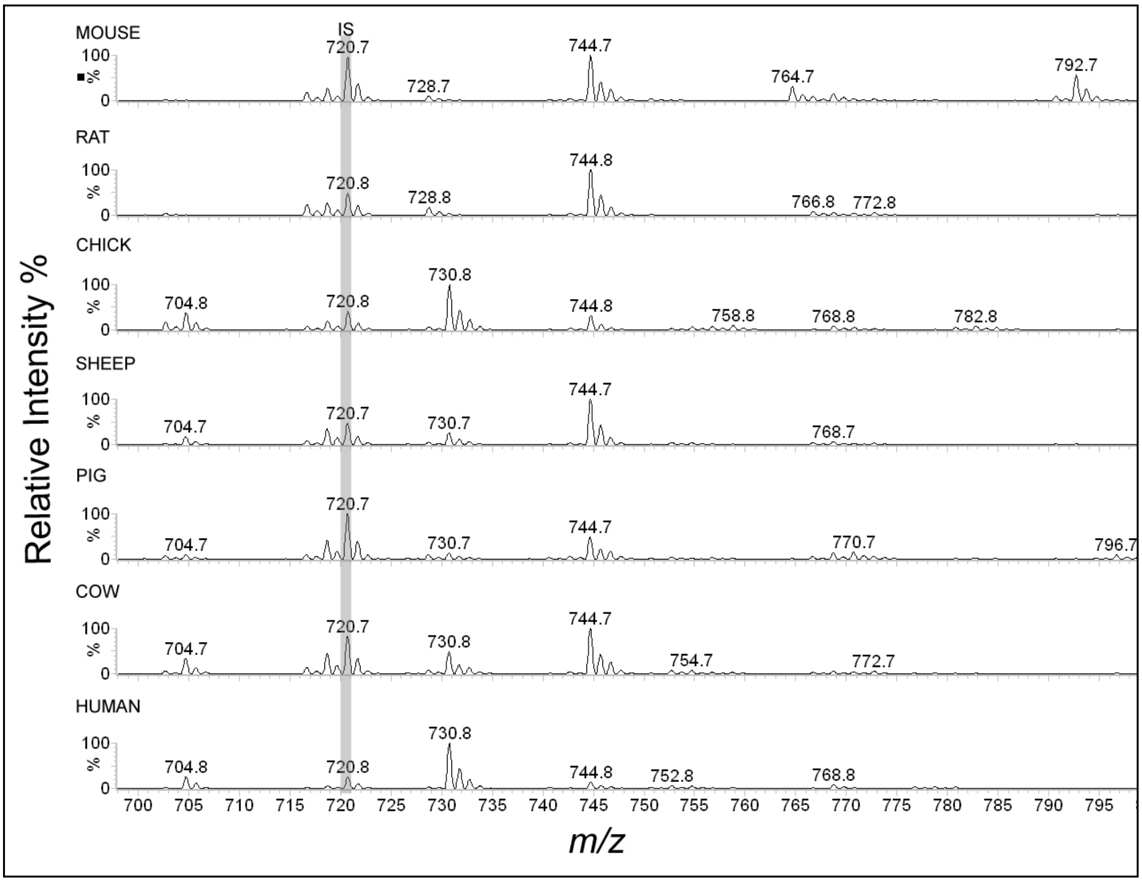

MOUSE
IS
720.7
744.7
728.7
764.7
792.7
RAT
744.8
720.8
728.8
766.8
772.8
CHICK
730.8
704.8
720.8
744.8
758.8
768.8
782.8
SHEEP
744.7
720.7
704.7
730.7
768.7
PIG
720.7
744.7
704.7
730.7
770.7
796.7
COW
720.7
744.7
704.7
730.8
754.7
772.7
HUMAN
730.8
704.8
720.8
744.8
752.8
768.8
100
%
0
Relative Intensity %
700 705 710 715 720 725 730 735 740 745 750 755 760 765 770 775 780 785 790 795
m/z

Figure 4

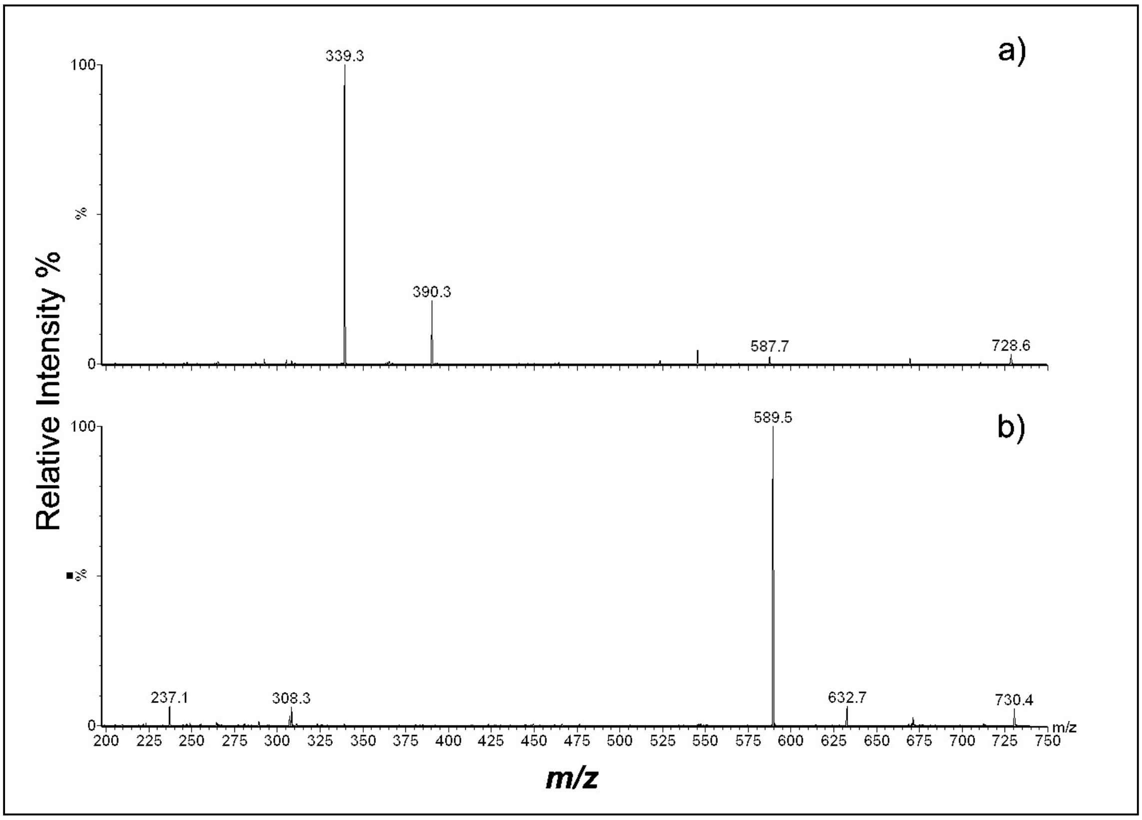

a)
b)
Relative Intensity %
100
0
%
339.3
390.3
587.7
728.6
589.5
237.1
308.3
632.7
730.4
200
225
250
275
300
325
350
375
400
425
450
475
500
525
550
575
600
625
650
675
700
725
750
m/z
m/z

Figure 5

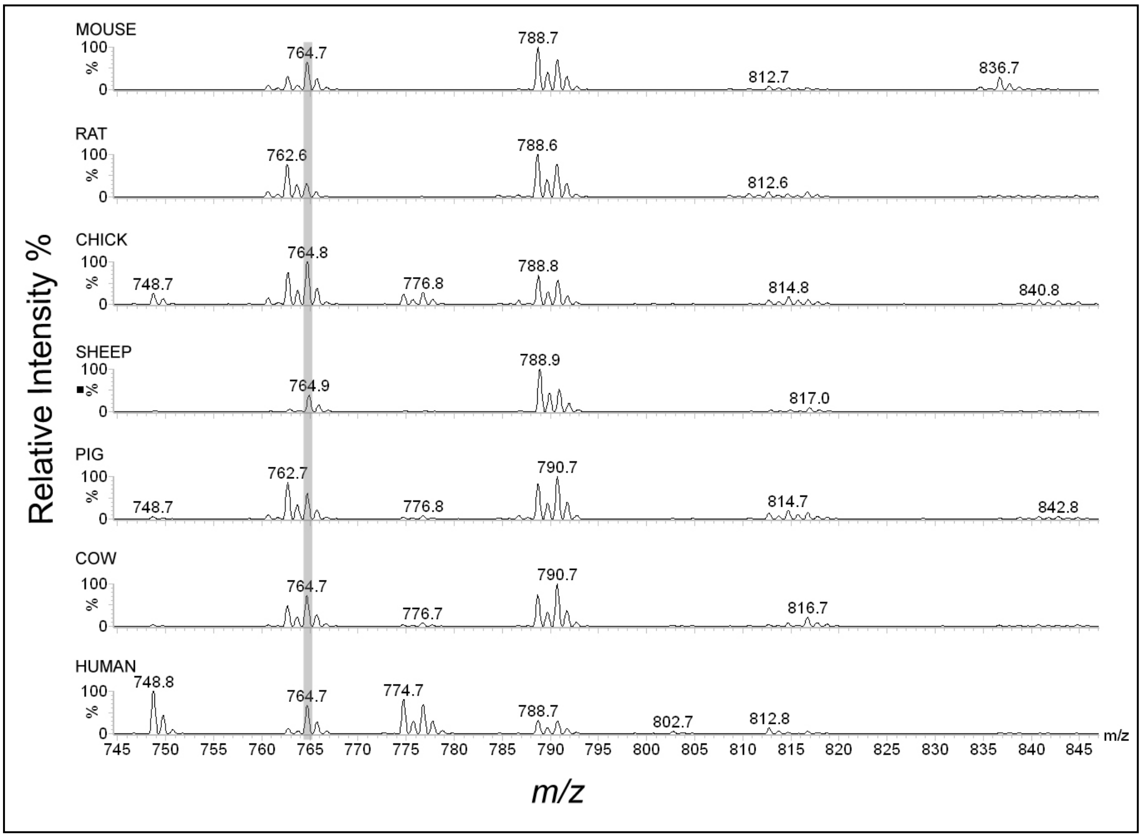

MOUSE
RAT
CHICK
SHEEP
PIG
COW
HUMAN
Relative Intensity %
m/z
764.7
788.7
812.7
836.7
762.6
788.6
812.6
748.7
764.8
776.8
788.8
814.8
840.8
764.9
788.9
817.0
748.7
762.7
776.8
790.7
814.7
842.8
764.7
776.7
790.7
816.7
748.8
764.7
774.7
788.7
802.7
812.8

Figure 6

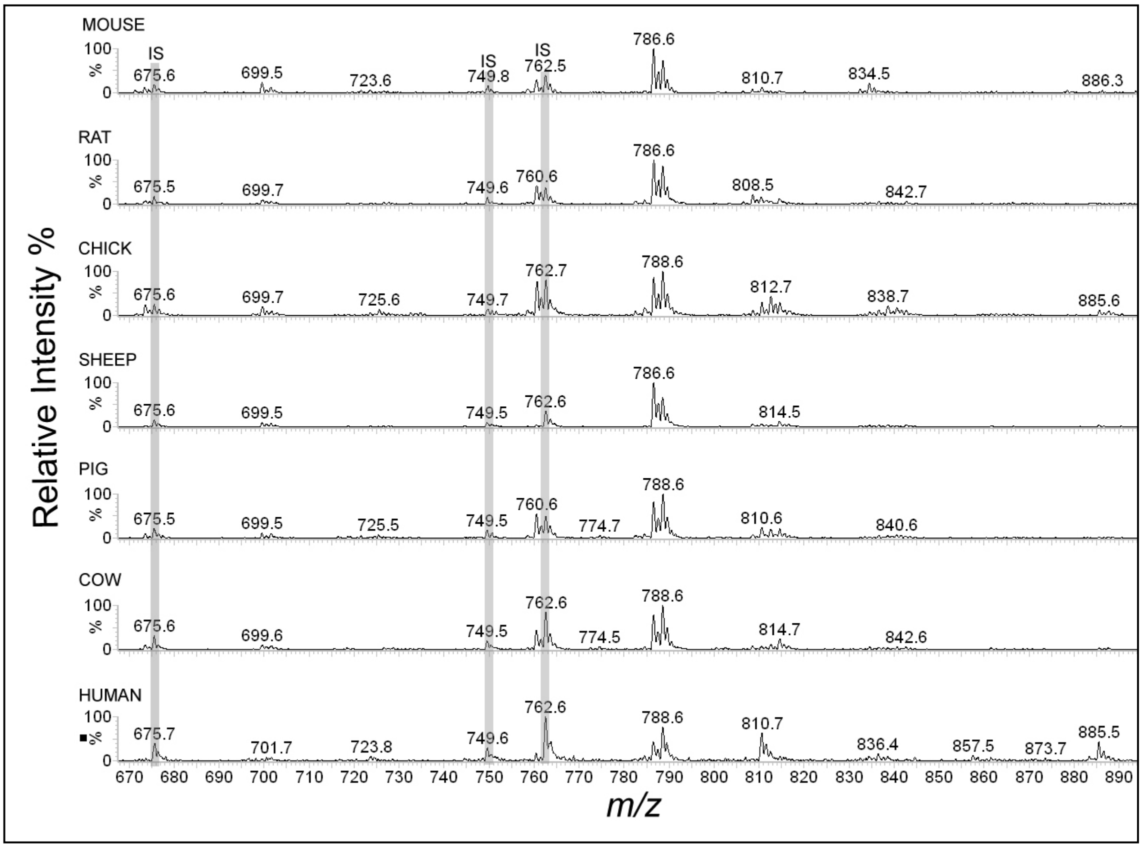

MOUSE
RAT
CHICK
SHEEP
PIG
COW
HUMAN
IS
786.6
788.6
762.6
749.5
675.6
Relative Intensity %
m/z

Figure 7

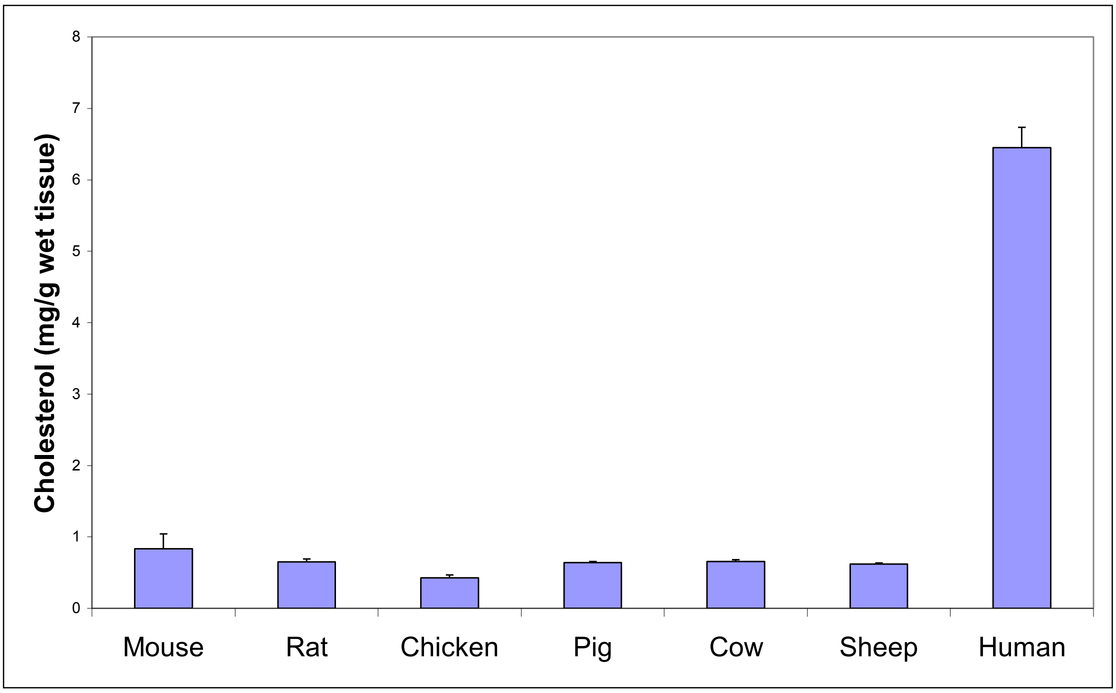

Cholesterol (mg/g wet tissue)
0
1
2
3
4
5
6
7
8
Mouse
Rat
Chicken
Pig
Cow
Sheep
Human

Figure 8

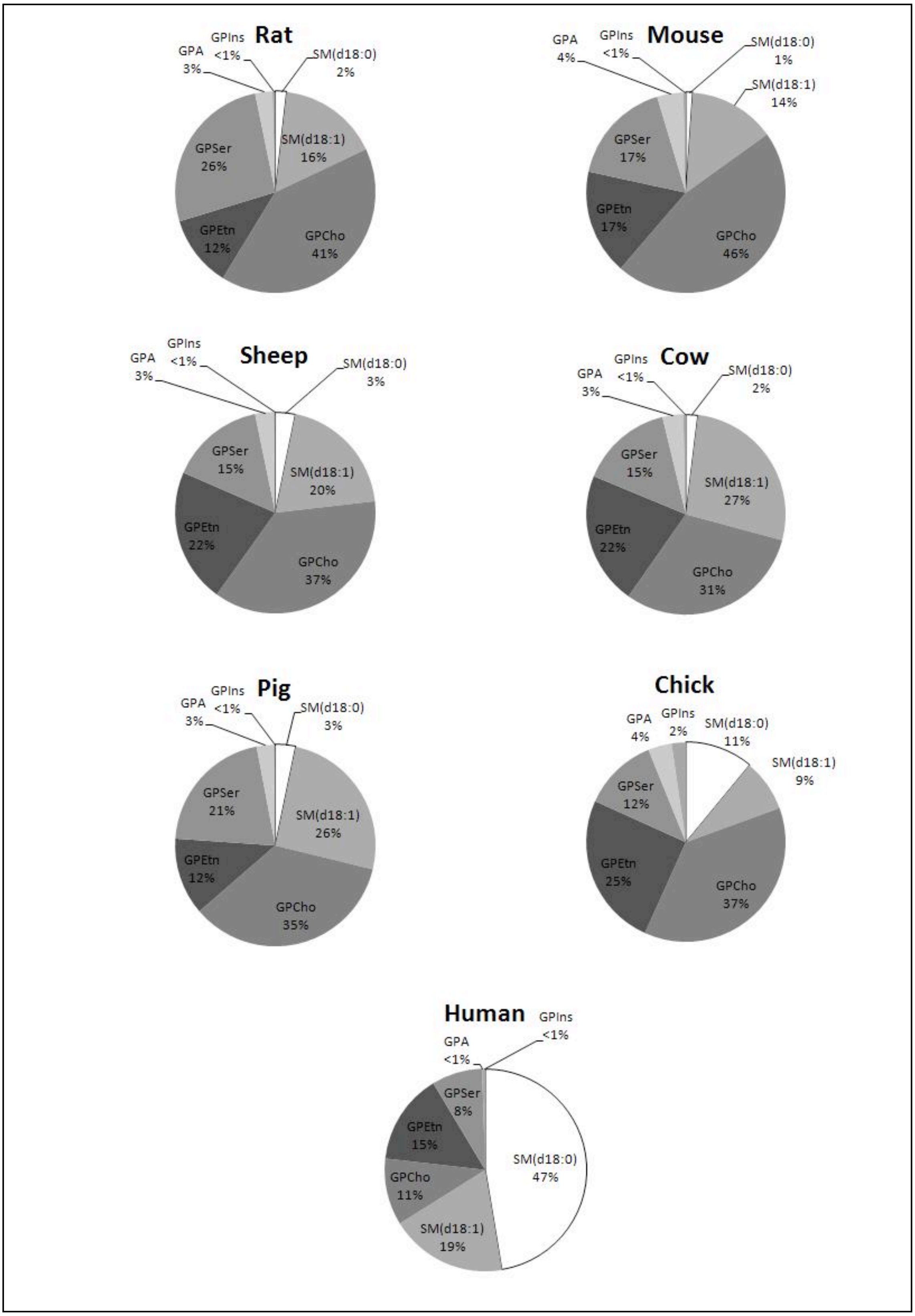

Rat
GPA 3%
GPIns <1%
SM(d18:0) 2%
SM(d18:1) 16%
GPCho 41%
GPEtn 12%
GPSer 26%
Mouse
GPA 4%
GPIns <1%
SM(d18:0) 1%
SM(d18:1) 14%
GPCho 46%
GPEtn 17%
GPSer 17%
Sheep
GPA 3%
GPIns <1%
SM(d18:0) 3%
SM(d18:1) 20%
GPCho 37%
GPEtn 22%
GPSer 15%
Cow
GPA 3%
GPIns <1%
SM(d18:0) 2%
SM(d18:1) 27%
GPCho 31%
GPEtn 22%
GPSer 15%
Pig
GPA 3%
GPIns <1%
SM(d18:0) 3%
SM(d18:1) 26%
GPCho 35%
GPEtn 12%
GPSer 21%
Chick
GPA 4%
GPIns 2%
SM(d18:0) 11%
SM(d18:1) 9%
GPCho 37%
GPEtn 25%
GPSer 12%
Human
GPIns <1%
GPA <1%
GPSer 8%
GPEtn 15%
GPCho 11%
SM(d18:1) 19%
SM(d18:0) 47%

Table Captions

Table 1. Abundant animal lens phospholipids. The two most abundant phospholipids within each phospholipid class. The mean relative abundance of each phospholipid as a percentage of that phospholipid class is listed in parentheses (N=4). SM(d18:1), sphingomyelin; SM(d18:0), dihydrosphingomyelin; GPCho, phosphatidylcholine; GPEtn, phosphatidylethanolamine; GPSer, phosphatidylserine; GPA, phosphatidic acid; GPIns, phosphatidylinositol; e, 1-*O*-alkyl ether; p, 1-*O*-alkenyl ether; X, not observed. A complete table, showing the identity and quantity of all phospholipids observed in each animal lens, is available as supplementary material.

Figure Captions

Figure 1

The concentration of each phospholipid class in human (60 years old) and animal lenses. SM(d18:1), sphingomyelin; SM(d18:0), dihydrosphingomyelin; GPCho, phosphatidylcholine; GPEtn, phosphatidylethanolamine; GPSer, phosphatidylserine; GPA, phosphatidic acid. Data are presented as mean + SEM (N=4)

Figure 2

Positive ion mass spectra of the phosphocholine cation (*m/*z 184) precursors showing the profile of all phosphocholine containing phospholipids, i.e. phosphatidylcholine (GPCho), sphingomyelin [SM (d18:1)] and dihydrosphingomyelin [SM (d18:0)], in a lens lipid extract from each species. The internals standards SM (d18:0/12:0) (*m/z* 649.7) and GPCho (19:0/19:0) (*m/z* 818.8) are highlighted.

Figure 3

Positive ion neutral loss mass spectra of 141Da (phosphoethanolamine) showing the phosphatidylethanolamine (GPEtn) profile in a lens lipid extract from each species. The internals standard GPEtn (17:0/17:0) (*m/z* 720.7) is highlighted.

Figure 4

The positive ion CID spectra of A) GPEtn (18:1p/18:1) in a pig lens crude lipid extract, and B) GPEtn (18:1e/18:1) in a 60 year old human lens crude lipid extract.

Figure 5

Positive ion neutral loss mass spectra of 185 Da (phosphoserine) showing the phosphatidylserine (GPSer) profile in a lens lipid extract from each species. The internals standard GPSer (17:0/17:0) (*m/z* 764.7) is highlighted.

Figure 6

Negative ion mass spectra of the dehydrated glycerphosphate anion (*m/*z 153) precursors in a lens lipid extract from each animal. This scan displays most anionic phospholipid classes, i.e, phosphatidic acid (GPA), Phosphatidylglycerol (GPGro), phosphatidylserine (GPSer) and phosphatidylinositol (GPIns). In this study however, this scan was only used for the identification and quantification of GPA and GPGro. The internals standards GPA (17:0/17:0) (*m/z* 675.6), GPGro (17:0/17:0) (*m/z* 749.6) and GPSer (17:0/17:0) (*m/z* 762.6) are highlighted.

Figure 7

A comparison of cholesterol concentration in lens lipids extracts. Data are presented as mean + SEM. (n=4)

Figure 8

The relative abundance of phospholipid classes in animal lens crude lipid extracts compared to 60 year old human. Each pie chart is constructed from the mean mole percent of each phospholipid class (n=4). SM(d18:1), sphingomyelin; SM(d18:0), dihydrosphingomyelin; GPCho, phosphatidylcholine; GPEtn, phosphatidylethanolamine; GPSer, phosphatidylserine; GPA, phosphatidic acid; GPIns, phosphatidylinositol.